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Therapeutics, Targets, and Chemical Biology

Cancer Research

Molecular Landscape of Acquired Resistance to Targeted Therapy Combinations in *BRAF*-Mutant Colorectal Cancer

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Abstract

Although recent clinical trials of BRAF inhibitor combinations have demonstrated improved efficacy in *BRAF*-mutant colorectal cancer, emergence of acquired resistance limits clinical benefit. Here, we undertook a comprehensive effort to define mechanisms underlying drug resistance with the goal of guiding development of therapeutic strategies to overcome this limitation. We generated a broad panel of *BRAF*-mutant resistant cell line models across seven different clinically relevant drug combinations. Combinatorial drug treatments were able to abrogate ERK1/2 phosphorylation in parental-sensitive cells, but not in their resistant counterparts, indicating that resistant cells escaped drug treatments through one or more mechanisms leading to biochemical reactivation of the MAPK signaling pathway. Genotyping of resistant cells identified gene amplification of *EGFR, KRAS*, and mutant *BRAF*, as well as acquired mutations in *KRAS, EGFR*, and *MAP2K1*. These mechanisms were clinically relevant, as we identified emergence of a *KRAS* G12C mutation and increase of mutant *BRAF* V600E allele frequency in the circulating tumor DNA of a patient at relapse from combined treatment with BRAF and MEK inhibitors. To identify therapeutic combinations capable of overcoming drug resistance, we performed a systematic assessment of candidate therapies across the panel of resistant cell lines. Independent of the molecular alteration acquired upon drug pressure, most resistant cells retained sensitivity to vertical MAPK pathway suppression when combinations of ERK, BRAF, and EGFR inhibitors were applied. These therapeutic combinations represent promising strategies for future clinical trials in *BRAF*-mutant colorectal cancer. *Cancer Res; 76(15); 4504–15. ©2016 AACR.*

Introduction

Activating mutations in the *BRAF* oncogene occur in approximately 7% of human malignancies, including 50%–60% of melanomas and 5%–8% of colorectal cancers (1). The most frequent *BRAF* mutation (V600E) affects the kinase domain, mimics BRAF phosphorylated state, and leads to constitutive activation of the protein (1). In colorectal cancer, *BRAF* mutations are associated with hypermethylated tumor subtypes and are linked with aggressive, less-differentiated, and therapy-resistant disease (2). Metastatic colorectal cancer (mCRC) patients with *BRAF* V600E–mutant tumors show poor sensitivity to the EGFR-targeted mAbs panitumumab and cetuximab and display poor prognosis with a median overall survival of only about 6 to 9 months (3).

BRAF V600E–mutant tumor types do not respond uniformly to BRAF-targeted therapy (4). Targeted inhibitors of mutant *BRAF* alone, or in combination with inhibitors of its downstream effector MEK, induce high response rates in *BRAF*-mutant melanoma (5, 6); in contrast, a phase I study of mCRC patients has shown that the BRAF inhibitor (BRAFi) vemurafenib has no clinical benefit when given as monotherapy (7). The molecular basis of this discrepancy has been partly explained by dissimilar EGFR expression levels between these two malignancies. Intrinsic resistance of colorectal cancer cells to BRAF or MEK-targeted agents is mediated by the release of a feedback loop, which activates EGFR signaling, leading to

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reactivation of MAPK signaling and often to upregulation of parallel PI3K–AKT pathways, triggering proliferation and survival (8–10). Melanomas are sensitive to BRAFi as they originate from the neural crest and do not express EGFR, making this feedback loop ineffective. On the other hand, colorectal cancers arise from epithelial cells in which EGFR is generally constitutively expressed.

These preclinical studies have provided the rationale for testing dual/triple vertical blockade of the MAPK pathway by targeting EGFR, BRAF, and MEK in *BRAF*-mutant mCRC patients. Combinations targeting EGFR, BRAF, and the prosurvival PI3K pathways are also being explored. Clinical objective responses have been seen in 20%–40% of patients treated with doublet or triplet combinatorial regimens (11–13).

Nevertheless, preliminary clinical evidence from phase Ib trials shows that responses are limited in duration (4, 11–16). The molecular basis underlying intrinsic or acquired resistance to these drug combinations in *BRAF*-mutant mCRC has not been comprehensively defined. The mechanisms by which cancer cells evade targeted therapies are usually molecularly heterogeneous, but they often converge downstream in the pathway, which was originally blocked by the targeted agent. For instance, cell lines and mCRC patients that become resistant to single-agent cetuximab or panitumumab show a variety of molecular mechanisms that converge in reactivating the MAPK pathway, including mutations in the drug-binding sites of *EGFR*, *RAS*/*RAF* amplification, or mutations, or genetic alterations leading to activation of alternative receptor tyrosine kinases (RTK) such as MET or HER2 (reviewed in ref. 17). Similarly, *BRAF*-mutant melanomas that become refractory to BRAF and/or MEK inhibitors (MEKi) also show a variety of molecular mechanisms leading to reactivation of MAPK and/or AKT signaling. These include increased expression of RTKs such as PDGFRβ, IGF-1R, and EGFR; overexpression of the COT kinase; mutation of MEK1 (*MAP2K1*) and MEK2 (*MAP2K2*) kinase; *MITF* or *NRAS* mutations; amplification, or alternative splicing of the *BRAF* gene; *CDKN2A* loss; or genetic alterations in the PI3K–PTEN–AKT pathway (reviewed in ref. 18).

On these premises, we hypothesized that heterogeneous genetic alterations leading to reactivation of the MAPK pathway could be responsible for acquired resistance to regimens cotargeting EGFR, BRAF, MEK, and PI3K in colorectal cancer patients, despite vertical pathway suppression at multiple key nodes. To perform a comprehensive assessment of the landscape of potential acquired resistance mechanisms, we cultured *BRAF*-mutant colorectal cancer cell lines in the presence of seven distinct clinically relevant combinatorial regimens until the emergence of resistant derivatives. These cell lines were subjected to genetic, biochemical, and functional analyses to identify molecular alterations underlying drug resistance. As *in vitro* modeling of acquired resistance in cancer cell models has proven effective in identifying resistance mechanisms that occur clinically (19–21), these findings may predict those mechanisms of resistance likely to arise in patients. These preclinical models also represent valuable tools for key functional studies aimed at identifying effective strategies to overcome drug resistance.

Materials and Methods

Generation of drug-resistant cell lines

WiDr parental cells were a gift from R. Bernards (The Netherlands Cancer Institute, Amsterdam, the Netherlands) in July 2011. HROC87 parental cells were shared by M. Linnebacher (University of Rostock, Rostock, Germany) in September 2011. VACO432 parental cells were obtained from Horizon Discovery in March 2011. The genetic identity of parental cell lines and their resistant derivatives was confirmed by short tandem repeat profiling (Cell ID System; Promega) not fewer than 2 months before drug profiling experiments. *BRAF*-mutant HROC87, VACO432, and WiDr cells were seeded in 100-mm dishes at a density of 5×10^6/plate and treated with drug combinations as indicated in Supplementary Table S1. Additional information is provided in Supplementary Materials and Methods.

Drug sensitivity assay

Cell proliferation and cytotoxicity were determined by cellular ATP levels (CellTiter-Glo Luminescent Assay; Promega) and DNA incorporation of a fluorescent cyanine dye (CellTox Green; Promega) after 72-hours drug treatment, respectively. Additional information is provided in Supplementary Materials and Methods and Supplementary Table S2.

Western Blot analysis

Protein quantification, SDS-PAGE, Western blotting, and chemiluminescent detection were performed as described previously (19). Detailed information is provided in Supplementary Materials and Methods.

Gene copy number analysis qPCR

Cell line DNA (10 ng) was amplified by quantitative PCR using the GoTaq QPCR Master Mix (Promega) with an ABI PRISM 7900HT apparatus (Applied Biosystems). *HER2*, *MET*, *EGFR*, *KRAS*, and *BRAF* gene copy number was assessed as described previously (19). Data were normalized to a control diploid cell line, HCEC (22), and expressed as the ratio between resistant and the corresponding parental cells. Primer sequences are reported in Supplementary Table S3.

FISH

Dual color FISH analysis was performed using Chr7q (7q11.21)/*BRAF* (7q34) probes, Chr7q/*EGFR* (7p12) probes, Chr12q (12q12)/*KRAS* (12p12.1) probes (Abnova), and all probe pairs were labeled with FITC and Texas Red, respectively. Details are provided in Supplementary Materials and Methods.

Candidate-gene mutational analysis

Cell line DNA was extracted by Wizard SV Genomic DNA Purification System (Promega) according to the manufacturer's directions. The following genes and exons were analyzed by automated Sanger sequencing by ABI PRISM 3730 (Applied Biosystems): *KRAS* (exons 2, 3, and 4), *NRAS* (exons 2 and 3), *BRAF* (exon 15), *EGFR* (exon 12), *MAP2K1* (exons 2 and 3), *MAP2K2* (exon 2). Primer sequences are listed in Supplementary Table S3.

Droplet digital PCR

Genomic DNA from colorectal cancer cells was amplified using ddPCR Supermix for Probes (Bio-Rad) using BRAF V600E assay (PrimePCR ddPCR Mutation Assay, Bio-Rad). Droplet digital PCR (ddPCR) was then performed according to the manufacturer's protocol and the results reported as percentage or fractional

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abundance of mutant DNA alleles to total (mutant plus wild-type) DNA alleles, as described previously (23).

Viral infection

The lentivirus production, cell infection, and transduction procedures were performed as described previously (24). WiDr cells were transduced with a lenti-control vector or a lentiviral vector carrying a mutated hBRAF V600E cDNA (a gift of Maria S. Soengas, CNIO, Madrid, Spain) or EGFR WT cDNA (a gift from Dr. C. Sun and Prof R. Bernards, The Netherlands Cancer Institute, Amsterdam, the Netherlands). VACO432 cells were transduced with a lentiviral vector carrying EGFR G465R–mutant cDNA (25).

Clinical samples

A chemorefractory mCRC patient was enrolled in the CMEK162×2110 clinical trial (clinical trial registration ID: NCT01543698) at Niguarda Cancer Center, Milan, Italy. The patient was treated with the BRAFi encorafenib (LGX818) in combination with the MEKi binimetinib (MEK162) from September 2013 to March 2014, obtaining a partial response in January 2014, followed by radiologic progression in March 2014. Blood samples from this patient were obtained at baseline (September 2013) and at progression (March 2014) through a separate liquid biopsy research protocol approved by the Ethics Committee at Ospedale Niguarda, Milan, Italy. The study was conducted according to the provisions of the Declaration of Helsinki, and the patient signed and provided informed consent before sample collection.

Next-generation sequencing analysis

Germline DNA was obtained from PBMC (Promega, ReliaPrep Tissue Kit), while cell free circulating DNA of tumor origin (ctDNA) was extracted from 2-mL plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions. Libraries were prepared with Nextera Rapid Capture Custom Enrichment Kit (Illumina Inc.), according to the manufacturer's protocol, as described previously (23). The custom-panel included the coding region of 226 genes, as detailed previously (23). Further details are provided in Supplementary Materials and Methods.

Bioinformatics analysis

NGS bioinformatics analysis was performed as described previously (23). Mutational analyses were the result of comparison between pre- and post-treatment samples. Details are provided in Supplementary Materials and Methods.

Results

Generation of models of acquired resistance to combinatorial therapies targeting EGFR–BRAF–MEK–PI3K

We selected three *BRAF* V600E–mutant colorectal cancer cell lines, HROC87, WiDr, and VACO432, which are resistant to single-agent BRAFi or MEKi, but sensitive to combined BRAFi/MEKi or their combinations with cetuximab (Supplementary Fig. S1). To gain a comprehensive understanding of potential therapeutic resistance mechanisms in *BRAF*-mutant colorectal cancer, cell lines were cultured until resistant derivatives emerged in the presence of seven different drug combinations currently being explored in clinical trials. The drugs included the BRAFi dabrafenib, encorafenib, and vemurafenib; the MEKi selumetinib and trametinib; the EGFR-targeted antibody cetuximab; and the selective PI3K-α inhibitor (PI3Ki) alpelisib (Fig. 1A). A total of eleven resistant cell line models were generated. Two independent resistant cell populations were obtained by growing VACO432 cells with vemurafenib and cetuximab (V+C) and these were therefore indicated as resistant A (R.A) and resistant B (R.B). Resistance to drug treatment was confirmed by cell viability assay comparing parental and resistant cell derivatives. All resistant cell models were clearly refractory at all drug concentrations tested (Fig. 1B).

Cells with acquired resistance to BRAF inhibitor combinations display biochemical reactivation of MAPK signaling

Prior studies indicate that tumors with acquired resistance to BRAF or EGFR-targeted agents in monotherapy maintain sustained levels of MEK/ERK or (occasionally) AKT phosphorylation even in the presence of drug (19, 26–29). We tested whether the same biochemical rewiring could occur in cells made resistant to combinations of therapies targeting EGFR-BRAF-MEK-PI3K. Amounts of total MEK, ERK, or AKT proteins were not substantially different between parental cells and their resistant counterparts. However, variation of their phosphorylation levels (pMEK, pERK, or pAKT) was evident after drug treatment. Some, but not all, resistant models displayed increased phosphorylation of AKT at Ser473 upon drug treatment. However, every resistant model showed sustained levels of ERK phosphorylation despite drug treatment, in stark contrast to parental cells in which robust inhibition of ERK phosphorylation was observed with all treatments (Fig. 2).

Overall, these analyses indicate that combinatorial EGFRi/BRAFi/MEKi/PI3Ki treatments abrogate ERK phosphorylation in parental-sensitive cells, but that their resistant counterparts can sustain MAPK signaling in the presence of these therapeutic combinations (Fig. 2).

Acquired molecular alterations in *BRAF*-mutant colorectal cancer cell lines confer resistance to BRAF inhibitor combinations

To identify likely candidate drug resistance mechanisms leading to biochemical reactivation of MAPK signaling, we focused our analysis on components of the MAPK pathway by performing copy number analyses of *HER2*, *EGFR*, *MET*, *KRAS* and *BRAF* and Sanger sequencing of the most pertinent exons of *EGFR*, *KRAS*, *NRAS*, *BRAF*, *MAP2K2*, and *MAP2K1*.

Quantitative PCR on genomic DNA extracted from resistant cells showed no changes in *HER2* or *MET* gene copy number while *EGFR*, *KRAS*, *or BRAF* gene copy number increased in three WiDr derivatives resistant to V+S, D+C or S+C, respectively (Fig. 3A). All gene amplifications were only found in the resistant cell populations and were confirmed by FISH analyses (Fig. 3B). Sanger sequencing of hotspot regions of *EGFR* (exon 12), *KRAS* (exons 2, 3, and 4), *NRAS* (exons 2 and 3), *BRAF* (exon 15), *MAP2K1* (exons 2 and 3), and *MAP2K2* (exon 2) revealed acquired gene mutations in eight cell lines, as summarized in Table 1. All resistant cell populations retained the original *BRAF* V600E mutation. All other mutations found in resistant cells were not detected in their parental counterparts by conventional Sanger sequencing.

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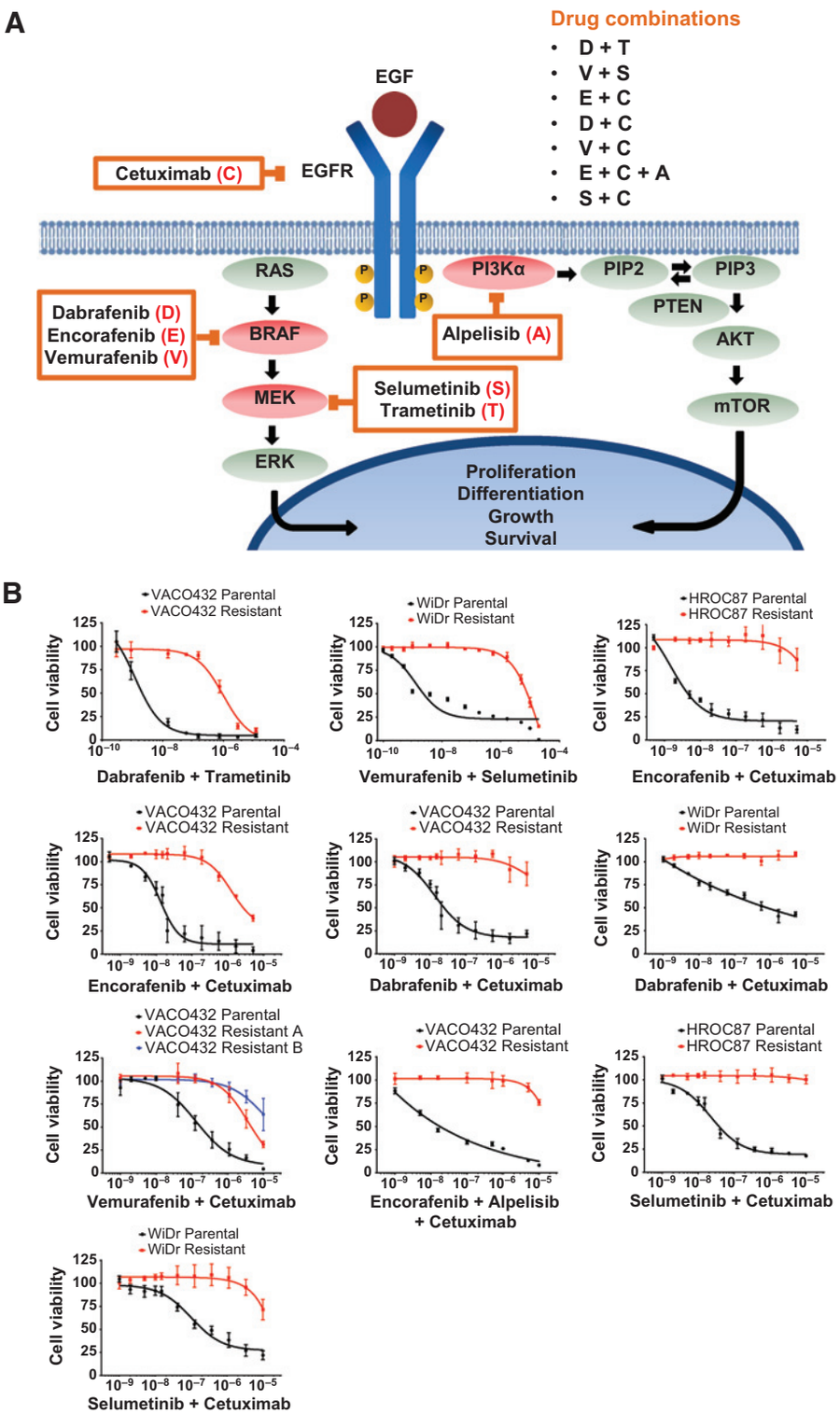

Figure 1.
Generation of *BRAF*-mutant colorectal cancer cells resistant to EGFR-targeted agent and BRAF/MEK or PI3K inhibitors. **A,** schematic representation of RAS/RAF/MEK and PI3K/AKT pathways. The orange boxes show the drugs used to generate resistant cell lines. List of the drug combinations used for generating resistant cell lines is shown at the top; all of these have been or are being evaluated in clinical trials. Drugs are abbreviated as follows: A, alpelisib (PI3K inhibitor, PI3Ki); C, cetuximab (EGFRi); D, dabrafenib (BRAFi); E, encorafenib (BRAFi); S, selumetinib (MEKi); T, trametinib (MEKi); V, vemurafenib (BRAFi). **B,** parental and resistant cells were treated for 72 hours with the indicated molar drug concentrations. Cetuximab and alpelisib were given at a constant concentration of 5 μg/mL and 100 nmol/L, respectively. In the vemurafenib and selumetinib combination, selumetinib was used at a constant concentration of 300 nmol/L.

Alterations in *KRAS* were the most common resistance mechanisms observed. Acquired *KRAS* mutations affecting exons 2 and 4 (G12D, G13D and A146T/V) were found in five different cell line models resistant to doublet BRAFi+EGFRi or triplet E+C+A. In one case, multiple *KRAS* mutations were concomitantly present in the resistant cell population, suggesting polyclonality. Prior functional studies in cell models have already demonstrated a causative role of

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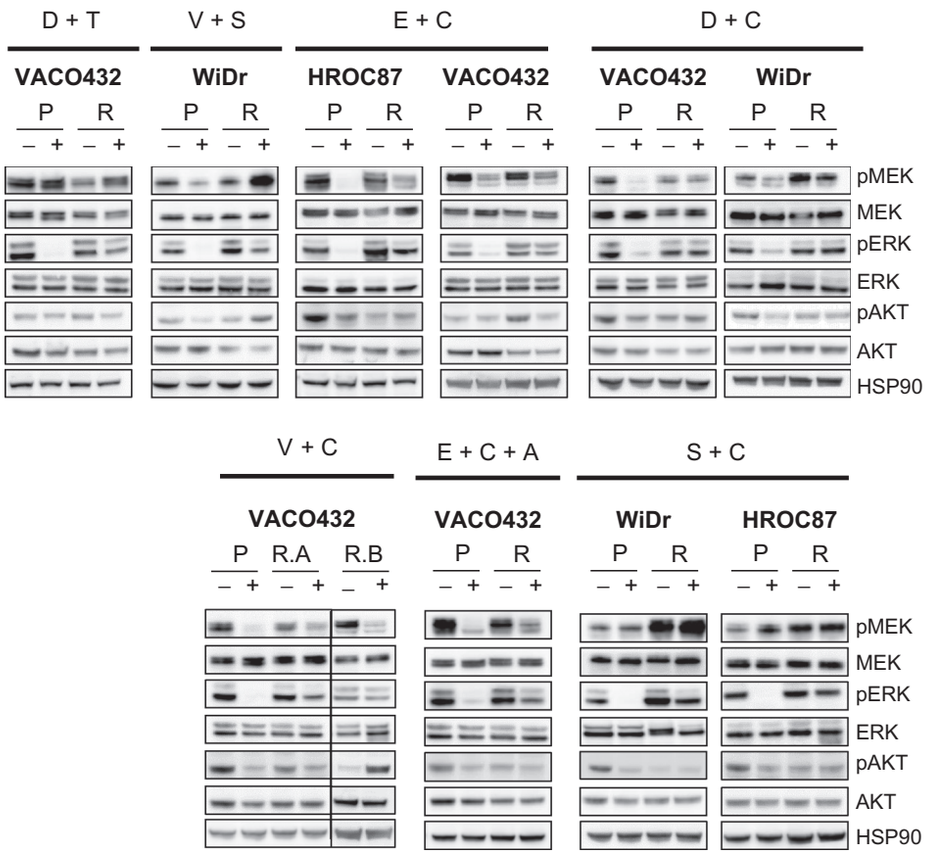

Figure 2.
Resistant cells maintain ERK1/2 phosphorylation after treatment. WiDr, VACO432, and HROC87 parental (P) and resistant (R) cells were treated with different drug combinations as indicated: cetuximab (C, 5 μg/mL); dabrafenib (D, 300 nmol/L); encorafenib (E, 400 nmol/L); alpelisib (A, 1 μmol/L); vemurafenib (V, 2 μmol/L); selumetinib (S, 1 μmol/L) and trametinib (T, 30 nmol/L). Drug treatment was given for 5 hours prior to protein extraction.

exon 2 *KRAS* mutations in driving resistance to BRAFi+EGFRi (30). Our data suggest that exon 4 *KRAS* mutations can also promote resistance. In addition, *KRAS* amplification was identified in WiDr resistant to BRAFi+EGFRi (D+C). *KRAS* amplification was found in the post-treatment biopsy of a colorectal cancer patient with acquired resistance to the combination of encorafenib and cetuximab (30). These findings suggest that the cell models generated in this work have the potential to recapitulate clinically relevant resistance mechanisms.

Increased *BRAF* gene copy number was seen in WiDr resistant to MEKi+EGFRi (S+C). Selective amplification of mutant *BRAF* V600E allele was previously identified in a *BRAF*-mutant colorectal cancer patient with acquired resistance to BRAFi+EGFRi (30), in colorectal cancer cell lines with secondary resistance to the MEKi selumetinib (31, 32), as well as in melanoma patients upon progression on the BRAFi vemurafenib (33), but not yet implicated in refractoriness to combined MEKi+EGFRi. To assess whether *BRAF* gene amplification had occurred in an allele-selective manner, we performed digital PCR analyses. WiDr parental cells carried 1 mutant and 3 wild-type alleles of *BRAF*, while their S+C resistant derivatives displayed a 9:1 mutant/wild-type ratio (Supplementary Fig. S2A). Western blot analysis with a diagnostic antibody specific for the V600E variant showed that the mutant protein was selectively overexpressed (Supplementary Fig. S2B). Finally, we validated that ectopic overexpression of mutant BRAF in WiDr parental cells can confer resistance to combined MEKi+EGFRi (Supplementary Fig. S2C and S2D).

Two different *MAP2K1* mutations leading to the V211D and L115P amino acid changes were identified in HROC87 and VACO432 resistant to MEKi+EGFRi (S+C) and BRAFi+MEKi (D+T), respectively. These mutations have previously been reported to confer resistance to MEK allosteric inhibitors in melanoma and colorectal cancer by preventing drug binding (27, 34), so they were not subjected to further functional validation.

Interestingly, amplification of *EGFR* was found in WiDr resistant to BRAFi+MEKi (V+S). Although EGFR signaling has been implicated in intrinsic resistance to BRAFi monotherapy in *BRAF*-mutant colorectal cancer (8, 9), *EGFR* gene amplification has not previously been established as a potential resistance mechanism in *BRAF*-mutant colorectal cancer. This result is consistent with previous observations that induction of EGFR protein expression can drive resistance to BRAFi or MEKi in melanoma (35). Ectopic overexpression of EGFR in WiDr parental cells was able to confer resistance to combined BRAFi+MEKi or BRAFi+EGFRi (Fig. 4A and B). Importantly, however, the triple combination of BRAFi+EGFRi+MEKi was able to restore sensitivity in resistant cells carrying *EGFR* amplification (Fig. 4C).

A single point mutation affecting the ectodomain of EGFR (G465R) was found in VACO432 V+C (R.B). Although this variant has previously been shown to disrupt receptor–antibody interaction, leading to cetuximab or panitumumab resistance in RAS/BRAF wild-type colorectal cancers (25), mutations affecting the EGFR ectodomain have not been reported previously as

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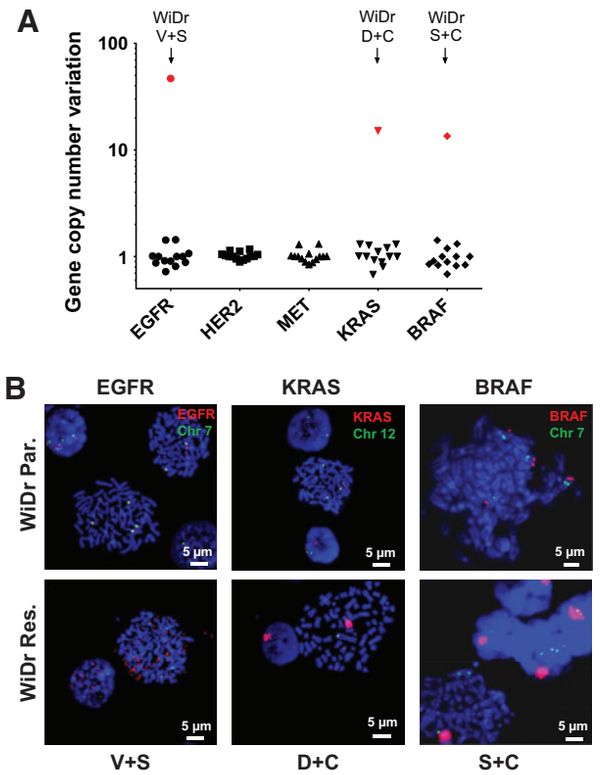

Figure 3.

EGFR, *KRAS*, and *BRAF* gene amplification confer acquired resistance to BRAF combination therapies. **A,** quantitative PCR for copy number evaluation of resistant cell lines with respect to their parental counterparts. WiDr V + S, D + C, and S + C–resistant lines displayed gene amplification of *EGFR*, *KRAS*, and *BRAF*, respectively. **B,** FISH analysis on chromosome metaphase spreads confirmed gene amplification. Cell nuclei were colored by DAPI, and FISH probes EGFR, KRAS, and BRAF were labeled with Texas Red (red signal) and chromosome 7 (Chr7) and 12 (Chr12) with FITC (green signal). *EGFR* gene amplification was found extrachromosomally as double minutes, while a focal intrachromosomal amplification of *KRAS* and *BRAF* loci could be identified.

potential resistance mechanisms in the context of *BRAF*-mutant tumors. To investigate the role of this mutation, we induced ectopic expression of *EGFR* G465R in VACO432 parental cells. Analysis of transduced cells indicated that the *EGFR* G465R mutation is able to sustain ERK phosphorylation and cell proliferation in the presence of combined V+C treatment (Fig. 4D and E). Cross-resistance to the combination of vemurafenib and panitumumab was seen. However, consistent with its known role in disrupting anti-EGFR Ab binding, the ability of the *EGFR* G465R mutation to promote resistance was specific to BRAFi + EGFRi combinations with anti-EGFR antibodies only, and kinase inhibition of EGFR by gefitinib was able to restore sensitivity in combination with BRAFi (Fig. 4F).

Clinical acquired resistance to combined therapy with BRAF and MEK inhibitors

Identification of clinical acquired resistance mechanisms to targeted therapy combinations was performed by genotyping of liquid biopsy samples. Plasma samples taken before treatment and after disease progression were collected from a patient with *BRAF* V600E–mutant colorectal cancer who had achieved a partial response on a RAF/MEK inhibitor combination (clinical trial registration ID: NCT01543698). ctDNA was extracted and subjected to molecular profiling by NGS analysis of a custom panel of 226 cancer-related genes (23). The analysis revealed that the percentage of reads carrying *TP53* p.R282W–mutated allele were consistent between the baseline and the progression plasma (Fig. 5), indicating similar ctDNA content in both samples. In contrast, the proportion of *BRAF* V600E–mutant reads at resistance was twice as much as those in the baseline, suggesting selective amplification of the *BRAF*-mutant allele. NGS analysis revealed concomitantly the emergence of a *KRAS* G12C allele, which was undetectable in the pretreatment sample. These results indicate that the mechanisms of resistance to target inhibitors identified in cell lines could faithfully recapitulate those found in clinical samples.

Overall, we observed that a diverse array of molecular mechanisms can drive acquired resistance to clinically relevant therapeutic combinations targeting the EGFR–BRAF–MEK–PI3K pathways in *BRAF*-mutant colorectal cancer. However, we also found that each of these heterogeneous resistance mechanisms converges on a common signaling output to promote resistance reactivation of MAPK signaling, suggesting that it may be possible to devise a universal targeted combination strategy capable of overcoming multiple resistance mechanisms.

Vertical combined suppression of the MAPK pathway has residual activity on drug-resistant cells

On the basis of our observations that all resistant cell models show persistent MAPK signaling activation (Fig. 2), we postulated that they could retain sensitivity to suppression of the pathway downstream. In this regard, previous data indicate that some melanomas with acquired resistance to BRAFi monotherapy can

Table 1. Molecular alterations acquired upon resistance to targeted therapy combinations in *BRAF*-mutant CRC cell lines

Drugs	Cell line	EGFR	KRAS	NRAS	MAP2K1	MAP2K2	BRAF Gene CNV
D + T	VACO432	WT	WT	WT	L115P	WT	None
V + S	WiDr	EGFR ampl.	WT	WT	WT	WT	None
E + C	HROC87	WT	G13D	WT	WT	WT	None
	VACO432	WT	A146T	WT	WT	WT	None
D + C	VACO432	WT	A146T	WT	WT	WT	None
	WiDr	WT	KRAS ampl.	WT	WT	WT	None
V + C	VACO432 R.A	WT	G12D	WT	WT	WT	None
	VACO432 R.B	G465R	WT	WT	WT	WT	None
E + C + A	VACO432	WT	A146V A146T	WT	WT	WT	None
S + C	HROC87	WT	WT	WT	V211D	WT	None
	WiDr	WT	WT	WT	WT	WT	600E ampl.

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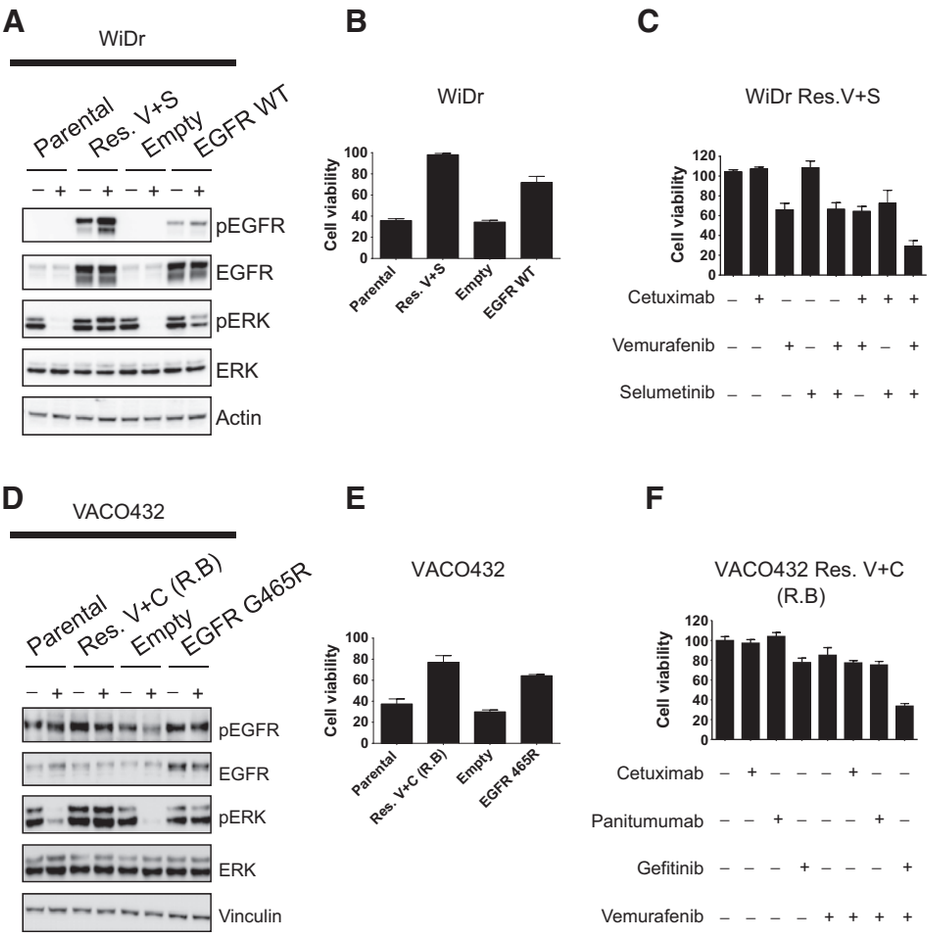

Figure 4.

EGFR amplification or ectodomain mutations play a causative role in acquired resistance to BRAF combination therapies. **A,** biochemical analyses of WiDr parental and V + S–resistant cell lines, and of WiDr cells transduced with either GFP cDNA or EGFR WT cDNA. Cells were treated with vemurafenib and selumetinib before protein extraction. Actin was used as a loading control. **B,** effect of vemurafenib (at the indicated molar concentrations) in combination with selumetinib (0.5 μmol/L) on the viability of WiDr cells transduced with EGFR WT cDNA. **C,** effect on cell viability of the addition of cetuximab to V + S treatment in WiDr-resistant cells carrying *EGFR* amplification. Cells were treated with vemurafenib (1 μmol/L), selumetinib (0.5 μmol/L), or cetuximab alone or with their combinations. **D,** EGFR and ERK expression and phosphorylation in VACO432 parental and resistant B cells, and in cells transduced with either GFP cDNA or EGFR G465R cDNA variants. VACO432 cells were treated with vemurafenib and cetuximab for 5 hours before protein extraction. Vinculin was used as a loading control. **E,** effect of vemurafenib (at the indicated molar concentrations) in combination with cetuximab (5 μg/mL) on the viability of VACO432 cells transduced with EGFR G465R cDNA. **F,** VACO432 with acquired *EGFR* G465R mutation upon treatment with vemurafenib and cetuximab retain sensitivity to vemurafenib and gefitinib treatment. All survival data were assessed by ATP content measurement after 72 hours of treatment. Data are expressed as average ± SD of two independent experiments.

benefit from additional treatment based on combined BRAFi and MEKi blockade (36). In addition, vertical triple blockade of EGFR + BRAF + MEK displayed the highest ability to suppress ERK phosphorylation in *BRAF* V600E colorectal cancer cells (37) and this combination has been shown to induce response rates of up to 40% in *BRAF*-mutant colorectal cancer patients (11). Similarly, previously published reports have documented promising preclinical activity of ERK inhibition in BRAFi or MEKi-resistant melanoma models (27, 38, 39) as well as in MEKi + BRAFi or BRAFi + EGFRi–resistant *BRAF*-mutant colorectal cancer cells (30). However, it has not yet been established whether ERK inhibitors might exhibit improved ability to overcome resistance when given as monotherapy, or in combination with BRAFi and/or EGFRi. Accordingly, we hypothesized that acquired resistance to BRAFi combinations could be overcome by more profound MAPK pathway suppression achieved by triplet combinations or by the incorporation of ERK inhibitor–based combinations. To test these hypotheses, the effect on viability was systematically tested across all resistant cell line models for all drug combinations used to generate resistant derivatives, as well as combinations incorporating the ERK inhibitor (ERKi) SCH772984 and the vertical cetuximab + dabrafenib + trametinib (BRAFi + MEKi + EGFRi) triplet combination (Fig. 6).

As expected, parental cell lines were highly sensitive to all drug treatments (Fig. 6). In general, resistant cell lines derived from one BRAFi + MEKi combination (D+T or V+S) showed cross-resistance to the other BRAFi + MEKi combination; and cell lines resistant to cetuximab in combination with encorafenib, dabrafenib, or vemurafenib were cross-resistant to other BRAFi + EGFRi combinations, irrespective of the specific drug used in the

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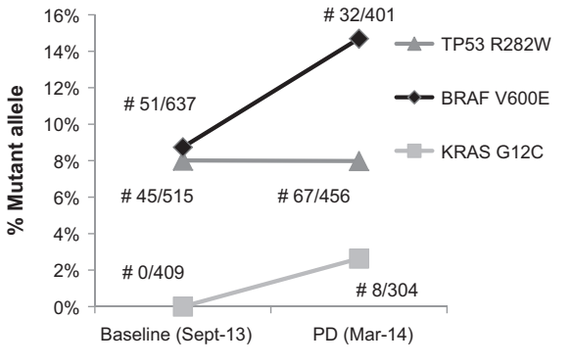

Figure 5.

Next-generation sequencing of ctDNA of a *BRAF*-mutant colorectal cancer patient at resistance to combined BRAF/MEK inhibition revealed an increase of *BRAF* V600E number of reads and the emergence of a *KRAS* G12C mutation. Data labels indicate number (#) of mutant reads over the total number of reads covering that position, detected by next-generation sequencing in ctDNA at baseline and resistance. PD, progressive disease.

selection protocol. This suggests that resistance mechanisms emerging under the selective pressure of these specific drug combinations are capable of conferring resistance to that class of inhibitors, and are unlikely to be related to any unique properties of the specific drugs used.

Interestingly, the addition of PI3Ki to BRAFi + EGFRi treatment did not robustly affect viability in any of the resistant cells relative to BRAFi + EGFRi alone. This finding is consistent with initial results of a clinical trial comparing encorafenib and cetuximab to encorafenib, cetuximab, and alpelisib, which have not demonstrated a clear benefit in response rate or progression-free survival with the addition of the PI3K inhibitor alpelisib (12, 40). In marked contrast, the triple combination of BRAFi + EGFRi + MEKi showed improved efficacy in many models relative to either BRAFi + EGFRi, BRAFi + MEKi, or MEKi + EGFRi alone. Finally, the addition of BRAFi and/or EGFRi to ERKi appeared to improve efficacy in some resistant models relative to ERKi alone, suggesting that ERKi may best be administered as part of therapeutic combinations in future clinical trials for *BRAF*-mutant colorectal cancer. Indeed, analysis of resistant cell lines indicated that ERK inhibition could induce cytotoxicity, which was further enhanced when combined with BRAFi and/or EGFRi (Supplementary Fig. S3A and S3B).

Discussion

Over the past few years, BRAF inhibitors have demonstrated striking clinical efficacy in patients with *BRAF*-mutant melanoma. However, BRAF inhibitors are not equally effective in other *BRAF*-mutant cancer histologies (4). Preclinical studies defining EGFR and MAPK pathway reactivation as key drivers of BRAFi resistance in *BRAF*-mutant colorectal cancer have provided the rationale for testing double or triple combinations of therapies targeting EGFR/BRAF/MEK/PI3K in this disease (11–13, 15, 16).

Unfortunately, while these approaches have led to improvements in response rate in *BRAF*-mutant colorectal cancer patients, preliminary clinical observations have indicated that, following an initial response, acquired resistance in *BRAF*-mutant colorectal cancer patients typically emerges after a few months of treatment (11–13, 15). The mechanisms underlying acquired resistance in *BRAF*-mutant colorectal cancer cells remain poorly characterized. In this study, we undertook a comprehensive effort to develop models of secondary resistance to a spectrum of seven clinically relevant combinatorial therapies to more robustly define the landscape of molecular mechanisms leading to acquired resistance in *BRAF*-mutant colorectal cancer. Our results indicate that the mechanisms leading to acquired resistance to these combinations can be genetically heterogeneous, but appear to converge on the reactivation of the MAPK signaling pathway at the biochemical level, suggesting that it might be possible to develop universal combination strategies capable of overcoming multiple resistance mechanisms. We acknowledge that no *in vivo* models were generated or assessed in this study, thus limiting our observations to cancer cell–autonomous drug resistance mechanisms. However, analyses of plasma samples at baseline and at acquired resistance to BRAF combinatorial therapy, in a *BRAF*-mutant colorectal cancer patient, revealed genetic alterations consistent with those identified in cell models, thus underscoring the clinical relevance of the broad panel of resistant lines generated in this work. As microenvironment and nongenomic mechanisms of drug resistance may also occur, future studies based on the analysis of BRAF-mutant murine models and patient samples will be needed to expand our knowledge on this aspect.

In our resistant cell line panel, we identified several novel mechanisms of acquired resistance not previously reported in *BRAF*-mutant colorectal cancer. In particular, we identified an *EGFR* G465R ectodomain mutation in a cell line with acquired resistance to the combination of a BRAFi and anti-EGFR antibody. While this mutation has been established as a mechanism of acquired resistance to anti-EGFR antibody monotherapy in RAS/BRAF wild-type colorectal cancer due to disruption of antibody binding (25), this class of mutations has not previously been implicated in *BRAF*-mutant colorectal cancer. Our observation warrants assessing for *EGFR* ectodomain mutations in *BRAF*-mutant colorectal cancer patients upon acquired resistance to BRAFi and anti-EGFR antibody combinations. Importantly, we found that a resistant model harboring this mutation retained sensitivity to BRAFi and an EGFR kinase inhibitor, as well as to downstream inhibitor combinations, such as BRAFi + MEKi. We also identified *EGFR* amplification as a novel potential mechanism of acquired resistance in *BRAF*-mutant colorectal cancer. Interestingly, unlike the *EGFR* ectodomain mutation, *EGFR* amplification conferred cross-resistance to BRAFi + EGFRi, BRAFi + MEKi, and MEKi + EGFRi combinations, likely as a consequence of increased EGFR signaling flux, and retained sensitivity only to the triple combinations of BRAFi + EGFRi + MEKi and ERKi + BRAFi + EGFRi. The finding that EGFR signaling leads to MAPK feedback reactivation and resistance during BRAFi monotherapy, but also can contribute to acquired resistance to MAPK combinatorial inhibition, highlights the central role of EGFR in the biology of *BRAF*-mutant colorectal cancer.

Molecular analyses of our resistance cell line panel also identified the presence of several resistance mechanisms previously identified in the setting of acquired resistance in *BRAF*-mutant colorectal cancer, including *KRAS* mutation or amplification, *BRAF* V600E amplification, and *MAP2K1* mutation (30), thereby underscoring the likely importance of these specific mechanisms within the spectrum of acquired resistance in *BRAF*-mutant colorectal cancer and supporting the likelihood that these specific alterations may be frequently observed in patients. This is also

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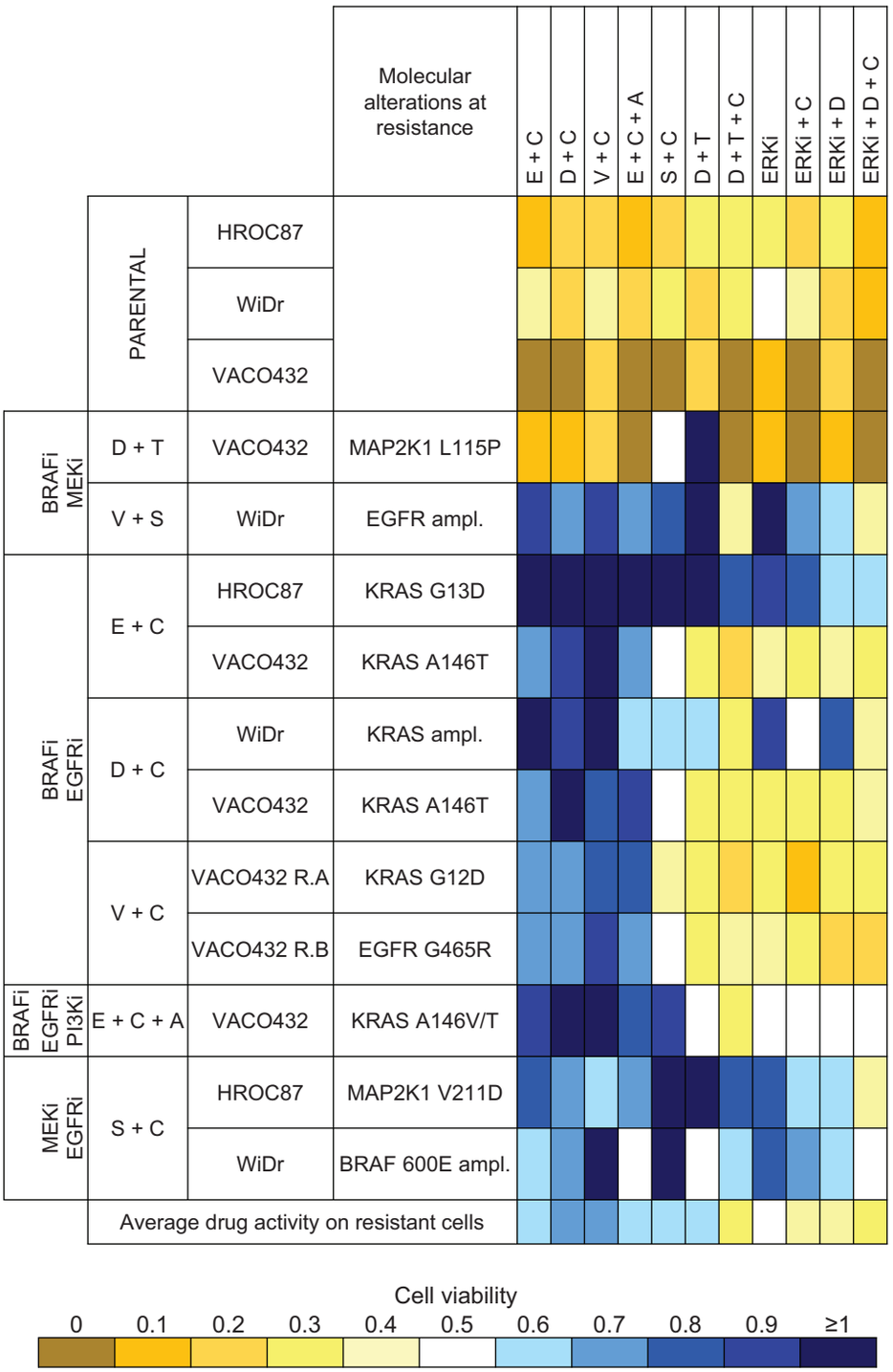

Figure 6.
Acquired resistance to target therapy combinations can be overcome by vertical MAPK pathway suppression. The viability of parental and resistant cell lines treated with different drug combinations targeting EGFR, BRAF, MEK, ERK, and PI3K was determined by ATP assay after 72 hours incubation. Relative survival was normalized to the untreated controls. Relative cell viability is depicted as indicated in the bottom color bar. Drugs were used at the concentrations listed in Supplementary Table S2. Results represent mean of at least two independent experiments, each performed in triplicate.

supported by the identification of two different genetic alterations identified at resistance to BRAF/MEK inhibition in plasma sample of a *BRAF*-mutant colorectal cancer patient, that is, the emergence of a *KRAS* mutation and a likely amplification of mutant *BRAF* V600E. In our resistant cell models, *KRAS* alterations were the most common resistance mechanism. The high prevalence of *KRAS* mutations in colorectal cancer and its role in resistance to anti-EGFR therapies underpin a central role for KRAS in this disease. Analysis by standard sensitivity sequencing has typically identified *KRAS* and *BRAF* mutations in a mutually exclusive fashion in colorectal cancer (41–43). To explain these observations, it has been suggested that concomitant oncogenic activation of KRAS and BRAF would be counter-selected during tumorigenesis, as it would result in activation of cell-cycle–inhibitory

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proteins of the Ink4/Arf locus, leading to oncogenic stress and senescence (44). Nevertheless, the use of more sensitive techniques, such as droplet digital PCR, has recently revealed that low-allele frequency *KRAS* mutations could coexist with *BRAF* V600E in colorectal cancer samples (7). These rare subclones may be present but might possess an unfavorable fitness compared to clones with only mutant *BRAF*. However, the selective pressure of BRAF-directed therapy may improve the proliferation rate of the double mutant clones while reducing the viability of cells bearing only mutant *BRAF*, thus driving outgrowth of resistant *BRAF*/*KRAS* double mutant clones. Indeed, a recent study analyzing tumor biopsies from *BRAF*-mutant colorectal cancer patients obtained prior to BRAF-directed therapy revealed that more than 50% bear low-frequency *KRAS* mutations (7). This finding might be explained by the "Big Bang" model (45), whereby tumors grow predominantly as a single expansion producing numerous intermixed subclones, where the timing of an alteration rather than clonal selection for that alteration is the primary determinant of its pervasiveness. Similarly, it is possible that some of the other common acquired resistance mechanisms we have observed in *BRAF*-mutant colorectal cancer, such as *BRAF* V600E amplification and *MAP2K1* mutation, may also pre-exist in rare tumor subclones. Indeed, we previously found that rare tumor cells with *BRAF* amplification could be identified in pretreatment tumor biopsies from *BRAF*-mutant colorectal cancer patients (32). Altogether, these observations suggest that *KRAS* as well as other resistance alterations could develop at an early stage of *BRAF*-mutant colorectal tumorigenesis, thus laying the seeds for the eventual emergence of acquired resistance. In a resistant cell model and in our patient, BRAF combinatorial therapies have resulted in the appearance of at least two concomitant resistance mechanisms. Indeed, the lower percentage of *KRAS*-mutant allele in comparison with the *TP53* founder mutation suggested that this variant may have been present in only a fraction of tumor cells distinct from the *BRAF* V600E–amplified subset. These data are consistent with previous reports in melanomas resistant to BRAFi, either as monotherapy or in combination with MEKi, in which multiple resistance mechanisms have been described to cooccur in individual patients (46, 47).

The observation that all resistance mechanisms identified in our cell panel converge to reactivate MAPK signaling has important clinical implications. As it may not be practical to design specific therapeutic strategies against each of the individual acquired resistance mechanisms observed in *BRAF*-mutant colorectal cancer, there would be clear clinical advantages to developing a more "universal" therapeutic strategy targeting a common signaling output that would be capable of overcoming a spectrum of potential resistance mechanisms. By systematically comparing multiple drug combinations designed to achieve more optimal MAPK pathway suppression across the molecular landscape of acquired resistance mechanisms in *BRAF*-mutant colorectal cancer, we were able to identify the most promising therapeutic candidates to overcome resistance. Although a few resistant cell lines showed only modest sensitivity to these combinations, suggesting the possibility that these models might harbor additional MAPK-independent resistance mechanisms, overall we observed that the combination of BRAFi + EGFRi + MEKi or ERKi in combination with BRAFi and/or EGFRi displayed superior activity across the vast majority of resistant models. Therefore, these combinations may represent the most promising strategies for evaluation in clinical trials for patients with *BRAF*-mutant colorectal cancer. Notably, the triple combination of BRAFi + EGFRi + MEKi is currently being evaluated in clinical trials, and preliminary results suggest improved response rate and progression-free survival compared with the individual doublet combinations (48), which is consistent with our findings, and suggests that improved activity against the common resistance mechanisms in *BRAF*-mutant colorectal cancer may account in part for the improved clinical efficacy observed.

Consistent with our findings, previously published reports have documented promising preclinical activity of ERK inhibition in BRAFi- or MEKi-resistant cell line models (27, 38, 39) and in MEKi + BRAFi and BRAFi + EGFRi–resistant *BRAF*-mutant colorectal cancer cells (30), supporting ERKi as key potential components of future clinical trial strategies for this disease. While it is likely that secondary mutations in ERK1/2 may limit the long-term efficacy of ERKi (49), it remains an important and unanswered question as to whether it is best to administer ERKi as monotherapy or whether ERKi might be more effective as part of drug combinations in *BRAF*-mutant colorectal cancer. Indeed, it is possible that ERK inhibition alone might trigger survival-promoting feedback loops through alternative pathways that might be optimally suppressed with therapeutic combinations. To help guide future clinical trial strategies, our study begins to address this critical question, and suggests that ERKi appears to be more effective against the spectrum of acquired resistance mutations in *BRAF*-mutant colorectal cancer when administered in combination with BRAFi and/or EGFRi inhibitors. In fact, the triplet combination of ERKi + BRAFi + EGFRi appeared to be the most effective combination strategy overall across our panel of resistant cell line models. Thus, our study suggests that initial clinical trials of ERKi in *BRAF*-mutant colorectal cancer patients should prioritize therapeutic combinations with BRAFi and EGFR inhibitors.

Disclosure of Potential Conflicts of Interest

G. Siravegna is a consultant/advisory board member for Trovagene. J. Tabernero is a consultant/advisory board member for Amgen, Boehringer Ingelheim, Roche, Sanofi, Symphogen, Taiho, Celgene, Chugai, Imclone, Lilly, Merck, Merck Serono, Millennium, and Novartis. J.A. Engelman reports receiving a commercial research grant from Novartis-Sponsored Research Agreement through Massachusetts General Hospital, AstraZeneca, and Sanofi-Aventis and is a consultant/advisory board member for Novartis, Sanofi-Aventis, Bristol Myer Squibb, Araxes, AstraZeneca, Chugai, Genentech, GSK, Merck, Roche, and Novartis. A. Bardelli is a consultant/advisory board member for Horizon Discovery, Biocartis, and Trovagene. S. Siena is a consultant/advisory board member for Amgen, Roche, Bayer, Eli Lilly, Sanofi, and Merrimack. R.B. Corcoran is a consultant/advisory board member for GSK, Merrimack Pharmaceuticals, Genentech, and Taiho Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Oddo, E.M. Sennott, L. Barault, G. Filiciotto, E. Elez, G. Crisafulli, G. Corti, G. Siravegna, J. Tabernero, A. Bardelli, R.B. Corcoran, F. Di Nicolantonio

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Cancelliere, R.B. Corcoran
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Molecular Landscape of Acquired Resistance to Targeted Therapy Combinations in *BRAF*-Mutant Colorectal Cancer

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